Steadman, David

Subject: 2nd eyes PP 8
Start Date: Friday, January 26, 2007
Due Date: Friday, February 02, 2007

Status: In Progress
Percent Complete: 0%

Total Work: 0 hours
Actual Work: 0 hours

Owner: Steadman, David
Requested By: Frederick, Elizabeth (Chugach)

Hi Dave,

Would you please review the following case(s) for the 2nd Pair of Eyes program.

AU	Serial Number	Status Date	Employee Name	Review Type	Reviewer
1614	10302171	1/19/2007	WEDDINGTON, KEVIN E	Red Folder	STEADMAN
1625	10477315	1/9/2007	OWENS, AMELIA A	Red Folder	STEADMAN

Thank you very much!

J. Mol. Microbiol. Biotechnol. (2001) 3(2): 295-300.

JMMB Research Article

Pyruvate Carboxylase is a Major Bottleneck for Glutamate and Lysine Production by *Corynebacterium glutamicum*

Petra G. Peters-Wendisch,[2] Bettina Schiel,[1] Volker F. Wendisch,[2] Efstratios Katsoulidis,[1] Bettina Möckel,[3] Hermann Sahm,[2] and Bernhard J. Eikmanns[1]*

[1]Dept. Microbiology and Biotechnology, University of Ulm, 89069 Ulm, Germany
[2]Institute of Biotechnology 1, Forschungszentrum Jülich, D-52425 Jülich, Germany
[3]Degussa Hüls AG, Abt. FA-FE-B, 33790 Halle, Germany

Abstract

Corynebacterium glutamicum **possesses both phosphoenolpyruvate carboxylase (PEPCx) and pyruvate carboxylase (PCx) as anaplerotic enzymes for growth on carbohydrates. To analyze the significance of PCx for the amino acid production by this organism, the wild-type *pyc* gene, encoding PCx, was used for the construction of defined *pyc*-inactive and *pyc*-overexpressing strains and the glutamate, lysine and threonine production capabilities of these recombinant strains of *C. glutamicum* were tested in comparison to the respective host strains. No PCx activity was observed in the *pyc*-inactive mutants whereas the *pyc*-overexpressing strains showed eight- to elevenfold higher specific PCx activity when compared to the host strains. In a detergent-dependent glutamate production assay, the *pyc*-overexpressing strain showed more than sevenfold higher, the PCx-deficient strain about twofold lower glutamate production than the wild-type. Overexpression of the *pyc* gene and thus increasing the PCx activity in a lysine-producing strain of *C. glutamicum* resulted in approximately 50% higher lysine accumulation in the culture supernatant whereas inactivation of the *pyc* gene led to a decrease by 60%. In a threonine-producing strain of *C. glutamicum*, the overexpression of the *pyc* gene led to an only 10 to 20% increase in threonine production, however, to a more than 150% increase in the production of the threonine precursor homoserine. These results identify the anaplerotic PCx reaction as a major bottleneck for amino acid production by *C. glutamicum* and show that the enzyme is an important target for the molecular breeding of hyperproducing strains.**

Introduction

Corynebacterium glutamicum and its subspecies *flavum* and *lactofermentum* are widely used in the industrial production of amino acids, particularly L-glutamate and L-lysine with estimated annual amounts of more than 800,000 and 350,000 tons, respectively (Leuchtenberger, 1996). In the past fifteen years, the biochemistry, physiology and the molecular biology of several amino acid biosynthesis pathways and recently also of central metabolic pathways have been intensively studied (reviewed in Sahm *et al.*, 1995; Krämer, 1996; Eggeling and Sahm, 1999). Single or combined overexpression or disruption of genes coding for (in some cases deregulated) enzymes involved in amino acid biosynthetic pathways enabled the redirection of the carbon flux towards a given amino acid in response to elevation or removal of the respective enzyme activity (e.g. Cremer *et al.*, 1991; Ikeda *et al.*, 1992; Katsumata and Ikeda, 1993; Reinscheid *et al.*, 1994; Morbach *et al.*, 1995; Eggeling *et al.*, 1998). Moreover, the metabolic fluxes within the central metabolism of *C. glutamicum* during growth on different carbon sources and during amino acid overproduction were analyzed in detail (e.g. Marx *et al.*, 1996, 1997; Dominguez *et al.*, 1998; Wendisch *et al.*, 2000).

The importance of precursor supply for amino acid production came in focus when Menkel *et al.* (1989) performed fumarate feeding experiments with *C. glutamicum* and found that the supply of oxaloacetate or aspartate might be a bottleneck for optimal lysine production. Based on carbon flux simulations it was proposed that the phosphoenolpyruvate carboxylase (PEPCx; see Figure 1) reaction is rate-limiting for lysine

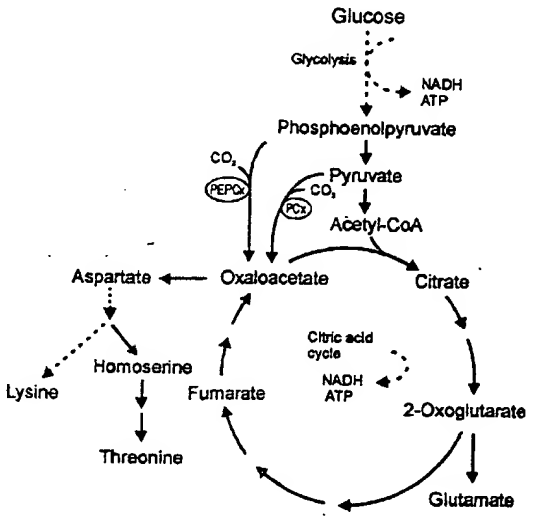

Figure 1. Diagramm of the central metabolism of *C. glutamicum* during growth on glucose and the relationship of the anaplerotic reactions of phosphoenolpyruvate carboxylase (PEPCx) and pyruvate carboxylase (PCx) to lysine, threonine and glutamate biosynthesis . Dotted arrows represent pathways consisting of several reactions, uninterrupted arrows represent single reactions.

Received September 11, 2000; revised October 16, 2000; accepted October 16, 2000. *For correspondence. Email bernhard.eikmanns@biologie.uni-ulm.de; Tel. +49 731 50 22707; Fax. +49 731 50 22719.

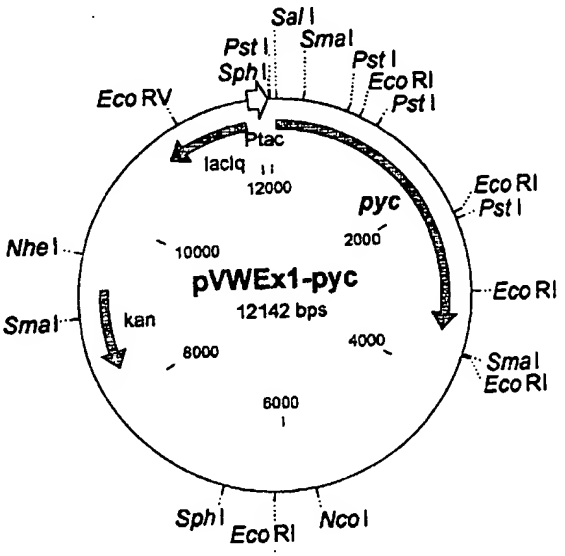

Figure 2. Restriction map of the *C. glutamicum-E. coli* vector pVWEx1-pyc. Abbreviations: kan, kanamycin resistance determinant; laclq, *lac* repressor gene $lacI^{Q}$; Ptac, *tac* promoter; *pyc*, *C. glutamicum* WT pyruvate carboxylase gene.

Table 1. Specific pyruvate carboxylase (PCx) activity in permeabilized cells of different *C. glutamicum* strains grown in glucose minimal medium in the presence and absence of 1 mM isopropyl-ß-D-thiogalactopyranoside (IPTG)

Strain	Specific activity (U / mg of dry weight)[a]	
	+ IPTG	- IPTG
C. glutamicum WT	20±3	19±4
C. glutamicum WT(pVWEx1)	22±3	20±4
C. glutamicum WT(pVWEx1-pyc)	202±19	26±13
C. glutamicum WTΔpyc	<1	<1
C. glutamicum DG52-5(pVWEx1)	8±2	6±2
C. glutamicum DG52-5(pVWEx1-pyc)	88±13	11±2
C. glutamicum DG52-5Δpyc	<1	<1
C. glutamicum DM368-3(pVWEx1)	10±1	11±3
C. glutamicum DM368-3(pVWEx1-pyc)	76±9	12±2

[a] Mean values ± standard deviations were obtained from at least three independent cultivations by at least two determinations per experiment.

production (Stephanopoulos and Vallino, 1991; Vallino and Stephanopoulos, 1993). However, overexpression and also inactivation of the PEPCx gene *ppc* in a lysine-producing strain resulted in only marginal or no effects on growth and lysine production (Cremer *et al.*, 1991; Peters-Wendisch *et al.*, 1993; Gubler *et al.*, 1994). Moreover, in temperature-triggered glutamate fermentations threefold amplification of PEPCx activity did not increase, but even slightly decreased the glutamate production (Delaunay *et al.*, 1999). All these results indicated that there is an alternative anaplerotic pathway present in *C. glutamicum* and that the PEPCx plays a minor role as anaplerotic enzyme.

Recently, we have been able to detect a pyruvate carboxylase (PCx; see Figure 1) in permeabilized cells of *C. glutamicum* and to isolate, characterize, and inactivate the respective *pyc* gene (Peters-Wendisch *et al.*, 1997, 1998). A defined PCx-negative mutant of *C. glutamicum* showed no growth on pyruvate or lactate, but did grow on glucose as sole carbon and energy source. In contrast, a defined PCx- and PEPCx-negative double mutant was unable to grow on glucose minimal medium (Peters-Wendisch *et al.*, 1998). These results indicated that in *C. glutamicum* no further anaplerotic enzymes for growth on carbohydrates exist apart from PEPCx and PCx and that these two enzymes can replace each other as anaplerotic enzyme for growth on glucose. By ^{13}C-labelling experiments with subsequent ^{13}C-NMR analyses, Petersen *et al.* (2000) obtained *in vivo* evidence for the simultaneous operation of PCx and PEPCx in glucose-growing cells of *C. glutamicum*, with the latter enzyme contributing only about 10% of the total oxaloacetate synthesis. In this study, we directly investigate the importance of PCx for glutamate, lysine, and threonine overproduction by genetic modification of the PCx activity in different *C. glutamicum* strains and analysis of the production performance of the recombinant strains in comparison to their parental strains.

Results

Overexpression and Inactivation of the PCx Gene in Different *C. glutamicum* Strains

C. glutamicum strains overexpressing the wild-type *pyc* gene were obtained by the transformation of *C. glutamicum* wild-type (WT), the lysine producer *C. glutamicum* DG52-5, and the threonine producer *C. glutamicum* DM368-3 with plasmid pVWEx1-pyc (Figure 2; for construction see Experimental Procedures). This plasmid carries the PCx gene *pyc* under control of the IPTG-inducible *tac* promoter. To prove the expression of the plasmid-borne *pyc* gene, PCx activities were determined in permeabilized cells of the pVWEx1-pyc carrying strains and compared with those of cells from the same strains carrying the vector pVWEx1 without insert. As shown in Table 1, the specific PCx activities of *C. glutamicum* WT(pVWEx1-pyc), *C. glutamicum* DG52-5(pVWEx1-pyc), and *C. glutamicum* DM368-3(pVWEx1-pyc) in cells grown in the presence of IPTG were eight- to elevenfold higher than in the strains carrying pVWEx1. In cells grown in the absence of IPTG, the PCx activity was independent of the presence or absence of the *pyc* gene within pVWEx1, showing that the *pyc*-overexpression in the pVWEx1-pyc carrying strains is due to IPTG-induced expression of the plasmid-borne *pyc* gene.

The construction and analysis of a defined PCx-negative derivative of *C. glutamicum* WT, strain WTΔpyc, was described previously (Peters-Wendisch *et al.*, 1998). This mutant was shown to be devoid of the 123 kDa biotinylated protein representing the PCx enzyme and, accordingly, devoid of any detectable PCx activity (Peters-Wendisch *et al.*, 1998 and Table 1). To obtain a PCx-negative mutant of the lysine producer *C. glutamicum* DG52-5, its chromosomal *pyc* gene was inactivated in the same way as described for the construction of *C. glutamicum* WTΔpyc. The resulting mutant *C. glutamicum*

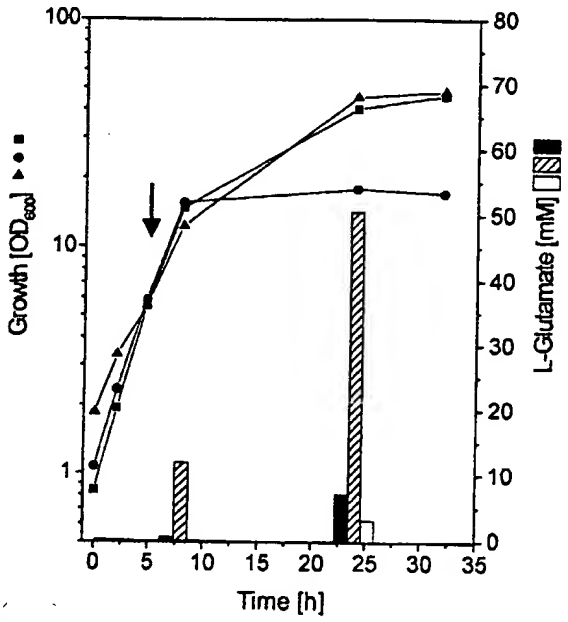

Figure 3. Growth and glutamate formation of *C. glutamicum* WT(pVWEx1) (▲,■), WT(pVWEx1-pyc)(●,▨), and WTpyc (■,□) on minimal medium containing glucose as carbon source and 0.5 mM IPTG. The arrow indicates the time when Tween 60 was added to the medium. The bars represent the glutamate concentrations determined in the three cultures at 8 and 24 hours after inoculation.

DG52-5Δpyc was tested for growth on different media and for PCx activity. The growth experiments revealed that, as in the case of *C. glutamicum* WTΔpyc, strain DG52-5Δpyc grew almost as well as the parental strain on minimal medium containing glucose but did not grow on minimal medium containing lactate or pyruvate as the sole carbon source. No PCx activity could be detected in permeabilized cells of *C. glutamicum* DG52-5Δpyc (Table 1), indicating that the *pyc* gene in this strain in fact is not functional.

Glutamate Production

To analyze the influence of the lack of PCx activity and of elevated PCx activity on glutamate production, we performed glutamate fermentations based on the addition of the fatty acid derivative Tween 60 (polyoxyethylene sorbitane monostearate) (Takinami *et al.*, 1965). Minimal glucose medium was inoculated with cells of *C. glutamicum* WT(pVWEx1), *C. glutamicum* WT(pVWEx1-pyc), and *C. glutamicum* WTΔpyc, and growth of the cultures and the glutamate concentration in the culture fluid analyzed. Without the addition of Tween 60, the pyc-mutant grew slightly slower (doubling time of 120 - 135 min) than *C. glutamicum* WT(pVWEx1) and the *pyc*-overexpressing strain (doubling times of about 95 - 105 min). However, all three strains grew to the same final optical density (OD_{600} = 45) and all three strains showed no significant glutamate secretion in a time period of up to 72 hours (<0.5 mM glutamate in the culture supernatant). When Tween 60 was added 5 hours after inoculation, the *pyc*-mutant and *C. glutamicum* WT(pVWEx1) showed the same growth behaviour as in the absence of Tween 60 whereas the *pyc*-overexpressing strain *C. glutamicum* WT(pVWEx1-pyc)

Table 2. Amino acid production by different derivatives of *C. glutamicum* DG52-5 and DM368-3.

Strain	Amino acid concentration (mM) SD in culture fluid[a]		
	Lysine	Threo-nine	Homo-serine
C. glutamicum DG52-5(pVWEx1)	34±1	<1	<1
C. glutamicum DG52-5(pVWEx1-pyc)	50±2	<1	<1
C. glutamicum DG52-5Δpyc	14±1	<1	<1
C. glutamicum DM368-3(pVWEx1)	n.d.[b]	8±1	6±1
C. glutamicum DM368-3(pVWEx1-pyc)	n.d.[b]	10±1	14±1

[a] Amino acid concentrations were determined after 48 h of cultivation in minimal medium containing glucose as a carbon source and in the presence of 1 mM isopropyl-ß-D- thiogalactopyranoside. Mean values standard deviations were obtained from nine independent cultivations by two determinations per experiment.
[b] n.d., not determined

reproducibly stopped growth about one doubling time (about 125 min) after addition of Tween 60 as exemplary shown in Figure 3. As also shown in Figure 3, *C. glutamicum* WT(pVWEx1) accumulated 7 to 8 mM glutamate, the *pyc*-overexpressing strain *C. glutamicum* WT(pVWEx1-pyc) about 50 mM glutamate, and the *pyc*-mutant *C. glutamicum* WTΔpyc about 3 mM glutamate within 19 hours after addition of Tween 60, i.e. 24 hours after inoculation. 72 hours after inoculation the glutamate concentrations in the respective cultures were 11 mM, 75 mM and 5 mM. Taking into consideration that *C. glutamicum* WT(pVWEx1-pyc) grew to a lower final optical density (OD_{600} = 18) than *C. glutamicum* WT(pVWEx1) (OD_{600} = 46) and WTΔpyc (OD_{600} = 48), the difference in the glutamate yield on biomass (glutamate produced per g of cells) between the *pyc*-overexpressing strain and the two other strains is even higher. The specific production rates within the first three hours after addition of Tween 60 were calculated to be 0.06, 1.56 and 0.02 mmol per g dry weight and hour for *C. glutamicum* WT(pVWEx1), *C. glutamicum* WT(pVWEx1-pyc) and *C. glutamicum* WTΔpyc, respectively. These results show that the capacity of *C. glutamicum* to produce glutamate is severely dependent on PCx activity.

To analyze the significance of the alternative anaplerotic enzyme, PEPCx, for Tween 60-dependent glutamate production by *C. glutamicum*, we also tested strains with altered PEPCx activities for their glutamate production capability. These strains were *C. glutamicum* WT-PP which possesses a chromosomal *ppc* disruption and shows no PEPCx activity (Peters-Wendisch *et al.*, 1993) and *C. glutamicum* WT(pMF1014-ppc) which displays more than 10-fold higher specific PEPCx activity when compared to the original host strain (Cremer *et al.*, 1991). Both recombinant strains showed the same growth behaviour as the parental WT strain and all three strains, WT, WT-PP and WT(pMF1014-ppc) showed identical glutamate formation, i.e. 7 to 9 mM glutamate 19 hours after the addition of Tween 60. These results show that PEPCx is dispensable for glutamate production and also that the level of PEPCx activity has no influence on glutamate production.

Lysine and Threonine Production

To analyze the consequences of *pyc*-overexpression and *pyc*-inactivation with respect to lysine production by *C. glutamicum*, the lysine-producing strain DG52-5 carrying the vector pVWEx1 without insert and the recombinant strains DG52-5(pVWEx1-pyc) and DG52-5Δpyc were grown on minimal medium plus glucose (40g/l) and the growth and the lysine concentration in the culture medium were analyzed. As in the case of the experiments with the WT strains, *C. glutamicum* DG52-5Δpyc grew slightly slower (doubling time of 145 - 155 min) than the original strain DG52-5 and the *pyc*-overexpressing strain DG52-5(pVWEx1-pyc)(doubling times of 125 - 135 min). All three strains grew to nearly the same final optical density of about 40. As shown in Table 2, the *pyc*-overexpressing strain accumulated approximately 50% more lysine and the *pyc*-mutant accumulated about 60% less lysine than the parental strain DG52-5 within 48 h of incubation. This result shows the significant influence of the PCx activity on the carbon flux to lysine.

To study the consequences of *pyc*-overexpression with respect to threonine production by *C. glutamicum*, the threonine- and homoserine-producing strain DM368-3 carrying pVWEx1 and the recombinant derivative DM368-3(pVWEx1-pyc) were cultivated on minimal medium plus glucose (40 g/l) and the threonine and homoserine concentrations in the culture fluid were analyzed. Both strains grew with identical doubling times and to the same final optical density. As shown in Table 2, overexpression of the *pyc* gene in *C. glutamicum* DM368-3 led to an only relatively slight increase in threonine production, however, to a more than 150% increase in the production of homoserine which is an intermediate of the threonine biosynthetic pathway. This result indicates that enhancing the anaplerotic activity by *pyc* overexpression is sufficient to increase the carbon flux into the threonine biosynthetic pathway, but that some step after homoserine formation limits the threonine production capability. As shown previously with recombinant isogenic *C. glutamicum* strains, the reactions converting homoserine to threonine by homoserine kinase and threonine synthase as well as the threonine export system (Palmieri *et al.*, 1996) might be limiting for high-level threonine production (Reinscheid *et al.*, 1994).

Discussion

The comparison of detergent-triggered glutamate production by the wild-type (WT) and the isogenic PCx-negative (WTΔpyc) and PEPCx-negative (WT-PP) mutants of *C. glutamicum* demonstrates that PCx is the major enzyme involved in fulfilling the anaplerotic demand for the production of glutamate. In the absence of PEPCx, the cells produced as much glutamate as in its presence showing that PEPCx is dispensable for glutamate production and that PCx can sustain the complete anaplerotic flux for this process. In the absence of PCx, the cells also produced glutamate, albeit to levels reduced to about 40%, indicating that PEPCx can partially complement the lack of PCx. As recent *in vivo* quantification of the carbon fluxes from PEP and pyruvate to oxaloacetate under non-production conditions revealed that PEPCx in *C. glutamicum* WT cells growing on glucose contributes only about 10% of the anaplerotic flux (Petersen *et al.*, 2000), it is likely that in the WT strain the contribution of PEPCx to glutamate production is also less than 40%. However, the relative *in vivo* activities of PCx and PEPCx and the carbon fluxes from PEP and pyruvate to oxaloacetate in the *C. glutamicum* WT strain under glutamate production conditions remain to be determined.

The significance of PCx and PEPCx for optimization of glutamate production by *C. glutamicum* can be deduced from the overexpression studies with the PEPCx gene *ppc* (Delauny *et al.*, 1999 and this study) and the PCx gene *pyc* (this study). An increase of PEPCx activity by genetic modification of the WT strain did not lead to an increase in glutamate production suggesting that PEPCx is down-regulated at higher glutamate production rates. PEPCx is effectively inhibited by low concentrations of aspartate (Ozaki and Shiio, 1969; Eikmanns *et al.*, 1989). It is conceivable that elevated PEPCx activity leads to higher intracellular oxaloacetate and aspartate concentrations and due to aspartate inhibition of PEPCx, no increase of the carbon flux from PEP to oxaloacetate would occur. That an increase of the carbon flux to oxaloacetate in principle is possible can be concluded from our experiments involving the *pyc*-overexpressing *C. glutamicum* derivative. The elevation of PCx activity led to an almost proportional increase in glutamate production indicating that the PCx enzyme is not inhibited or downregulated and that glutamate production was directly limited by the anaplerotic flux from pyruvate to oxaloacetate under the conditions employed.

Glutamate production by *C. glutamicum* can be triggered by various methods, i.e. the addition of antibiotics (Nunheimer *et al.*, 1970) or detergents (Takinami *et al.*, 1965), by applying a temperature-shift (Momose and Takagi, 1978) or biotin limitation (Shiio *et al.*, 1962; Hoischen and Krämer, 1990; Gutmann *et al.*, 1992). The mechanism of the induction of glutamate formation by either method is not yet understood completely, although it seems clear that glutamate excretion is always preceeded by a membrane alteration (Hoischen and Krämer, 1990; Krämer, 1996). As shown by Peters-Wendisch *et al.* (1997), biotin limitation results in reduced levels of PCx and of the biotinylated subunit of acyl-CoA carboxylase which is required to provide the building blocks for lipid biosynthesis and thus for the cytoplasmic membrane. The pleiotropic effects of biotin limitation make it difficult, if not impossible, to distinguish the role of either of the two biotin-containing proteins in *C. glutamicum* for glutamate production under these conditions. However, Delauny *et al.* (1999) recently interpreted a reduction in glutamate production when triggered by both temperature-shift and biotin limitation as compared to triggering by temperature-shift alone also as an indirect indication for an important role of PCx.

The importance of PCx activity for lysine production by coryneform bacteria has previously been postulated based on several lines of indirect experimental evidence. (i) Tosaka *et al.* (1979) showed that an excess of biotin in the medium has a promotive effect on lysine production by *Brevibacterium lactofermentum* and postulated this effect to be due to activation of a biotin-dependent pyruvate carboxylase. (ii) We showed that the lack of PEPCx and

the glyoxylate cycle did not impair lysine production by *C. glutamicum* (Peters-Wendisch *et al.*, 1996). The fact that the PEPCx-deficient *C. glutamicum* strains exhibited a higher biotin demand than the respective parental strains (Peters-Wendisch *et al.*, 1997) pointed to a biotin-containing pyruvate carboxylase as anaplerotic enzyme for lysine production. iii) Labeling experiments with a pyruvate kinase-deficient mutant and a pyruvate kinase- and PEPCx-deficient double mutant growing on gluconate plus ^{13}C-pyruvate gave indications for a pyruvate-carboxylating activity contributing approximately 90% of the oxaloacetate synthesis under these conditions (Park *et al.*, 1997). The reduced lysine productivity of the defined PCx-deficient strain *C. glutamicum* DG52-5 and the enhanced lysine productivity of the *pyc*-overexpressing strain as shown in this study unequivocally show that PCx activity in fact is a bottleneck for lysine production by *C. glutamicum*. As in the case with glutamate production, only about 40% lysine was produced by the *C. glutamicum* strain lacking PCx whereas *C. glutamicum* strains lacking PEPCx showed identical capacity for lysine production as the parental strains (Peters-Wendisch et al., 1993; Gubler *et al.*, 1994). Also,the overexpression of the PEPCx gene (*ppc*) had only a marginal effect on lysine productivity of *C. glutamicum* DG52-5 (Cremer *et al.*, 1991), corroborating the dissimilar importance of PCx and PEPCx for amino acid production. However, although we observed a positive effect of the increased PCx activity on lysine production, the increase of *pyc* expression did not correlate quantitatively with the increase of lysine production. The result suggests that after having overcome PCx as bottleneck a further metabolic function becomes limiting for lysine production. This limitation might take place in the lysine biosynthetic pathway, e.g. at the level of dihydrodipicolinate synthase which has been shown to be important for lysine production (Eggeling *et al.*, 1998). Alternatively, the lysine export driven by the lysine transporter LysE (Broer and Krämer, 1991; Vrljic *et al.*, 1996; Vrljic *et al.*, 1999) might be limiting for high-level lysine production by the *pyc*-overexpressing strain.

Our experiments directly addressed the significance of PCx activity for glutamate and lysine production by *C. glutamicum* in shake flask batch cultures. To our knowledge, the results described here for the first time prove that the anaplerotic flux sustained by PCx is a major bottleneck for the microbial production of primary metabolites. Thus, PCx is an important target for breeding hyperproducing strains to be used in large scale fermentation processes such as the industrial glutamate and lysine production.

Experimental Procedures

Bacteria, Plasmids, and Culture Conditions

The wild-type (WT) strain of *Corynebacterium glutamicum* ATCC 13032, the lysine producer *C. glutamicum* DG52-5 (Cremer *et al.*, 1988) and the threonine producer *C. glutamicum* DM368-3 (Eikmanns *et al.*, 1991) were employed for the construction of the recombinant strains used in this study. Additionally, we used the defined PCx-negative mutant of the WT strain *C. glutamicum* WTΔpyc (Peters-Wendisch *et al.*, 1998), the defined PEPCx-negative mutant of the WT strain *C. glutamicum* WT-PP (Peters-Wendisch *et al.*, 1993), and *C. glutamicum* WT(pMF1014-ppc) which has previously been shown to overexpress the PEPCx gene (Cremer *et al.*, 91). The PCx-negative *C. glutamicum* DG52-5Δpyc was constructed by the *pyc* gene replacement method described previously in detail for the construction of *C. glutamicum* WTΔpyc (Peters-Wendisch *et al.*, 1998). For plasmid construction, *E. coli* DH5 (Hanahan, 1985) was used. The plasmids employed were pK19mobsacB-pyc (Peters-Wendisch *et al.*, 1998) for the construction of the PCx-negative mutant of *C. glutamicum* DG 52-5, and pVWEx1 and pVWEx1-pyc for construction of the *pyc*-overexpressing strains of *C. glutamicum*. The expression plasmid pVWEx1 was constructed by ligating the 2.4 kb *ClaI-DraI* fragment from plasmid pEKEx2 (Eikmanns *et al.*, 1994) into the *BamHI-KpnI* restricted and blunt-ended *C. glutamicum/E. coli* shuttle vector pJC1 (Cremer *et al.*, 1988). The fragment cloned into pJC1 carries the *E. coli lac* repressor gene *lacI^Q*, upstream thereof and in the opposite orientation, the IPTG inducible *tac* promoter followed by a multiple cloning site. For construction of plasmid pVWEx1-pyc (Figure 2), the promoterless PCx gene was amplified from plasmid pEK0pyc (Peters-Wendisch *et al.*, 1998) using Vent DNA polymerase (NEB) and primers A (5'-GCTTCTAGACAGTGACTGCTATCACCCTTG-3') and C (5'-TGGAGATCTCGAATCAGACCAAATCC-3'). Primer A corresponds with its 21 nucleotides at the 3'-end (underlined) to nucleotides 112 to 132 in the *pyc* sequence deposited at the EMBL data base (accession number Y09548) and thus starts 4 nucleotides downstream of the transcriptional start site of the original gene. Primer C corresponds with its 17 nucleotides at the 3'-end to nucleotides 3682 to 3666 in the deposited *pyc* sequence. Both primers contain a *XbaI* site at their 5'-end. After generation of the *pyc* fragment by PCR, it was restricted with *XbaI*, purified, and ligated into the single *XbaI* site within the multiple cloning site of pVWEx1.

All *C. glutamicum* strains were pre-cultured on LB complex medium (Sambrook *et al.*, 1989) with kanamycin (50 g/ml) when appropriate. The minimal medium used for growth of and amino acid production by *C. glutamicum* was described previously (Eikmanns *et al.*, 1991) and contained glucose (40g/l) and 2 mg biotin/l. The cultures (60-ml in 500-ml baffled Erlenmeyer flasks) were inoculated to give an optical density at 600 nm (OD_{600}) of about 1 and then incubated aerobically at 30°C on a rotary shaker at 140 rpm. In glutamate fermentation experiments, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the cultures after 4 hours and 1.5 g Tween 60 (obtained from Sigma-Aldrich, Deisenhofen, Germany) (25 mg/ml), pre-warmed to 50°C, was added after 5 hours.

Preparation of DNA, Transformation, and DNA Manipulations

Plasmids from *E. coli* were isolated as described by Birnboim (1983). Plasmids from *C. glutamicum* were isolated by the same method except that the cells were preincubated with lysozyme (15 mg/ml, 1h, 37°C). *E. coli* was transformed by the $CaCl_2$ method (Sambrook *et al.*, 1989), *C. glutamicum* by electroporation (Liebl *et al.*, 1989). All restriction enzymes, T4 DNA ligase, Klenow polymerase and calf intestine phosphatase were obtained from Roche (Mannheim, Germany). Vent DNA polymerase was purchased from New England Biolabs (Schwalbach, Germany).

PCx Assays

PCx activity was determined in permeabilized cells of *C. glutamicum* using the glutamate-oxaloacetate-transaminase-coupled discontinuous assay described previously (Peters-Wendisch *et al.*, 1998). The aspartate formed was quantified by reversed-phase HPLC.

Amino Acid Analysis

For the analysis of amino acid accumulation in the culture fluid, aliquots were withdrawn and the cells were removed by centrifugation (5 min at 13,000 x g). Amino acids were analyzed as *ortho*-phtaldialdehyde derivatives by reversed phase chromatography as described previously (Schrumpf *et al.*, 1991).

Acknowledgements

We thank Eva Krings, Silke Dlugai, Petra Dangel and Caroline Kreutzer for excellent technical assistance. This work was supported by grant BIO 4-CT96-0145 within the 4th Framework Programme of the EC. The authors also acknowledge the support from Degussa-Hüls.

References

Birnboim, H. C. 1983. A rapid alkaline extraction method for the isolation of plasmid DNA. Methods Enzymol. 100: 243-255.

Broer, S., and Krämer, R. 1991. Lysine excretion by *Corynebacterium glutamicum*. Eur. J. Biochem. 202: 131-135.

Cremer, J., Eggeling, L., and Sahm, H. 1991. Control of the lysine biosynthetic sequence in *Corynebacterium glutamicum* as analyzed by overexpression of the individual corresponding genes. Appl. Environm. Microbiol. 57: 1746-1752.

Cremer, J., Treptow, C., Eggeling, L., and Sahm, H. 1988. Regulation of the enzym,es of lysine biosynthesis in *Corynebacterium glutamicum*. J. Gen. Microbiol. 134: 3221-3229.

Delaunay, S., Uy, D., Baucher, M.F., Engasser, J.M., Guyonvarch, A., and

Goergen, J.L. 1999. Importance of phosphoenolpyruvate carboxylase of *Corynebacterium glutamicum* during temperature triggered glutamic acid fermentation. Metab. Engin. 1: 334-343.

Dominguez, H., Rollin, A., Guyonvarch, A., Guerquin-Kern, J.L., Cocaign-Bousquet, M., and Lindley, N.D. 1998. Carbon flux distribution in the central metabolic pathways of *Corynebacterium glutamicum* during growth on fructose. Eur. J. Biochem. 254: 96-102.

Eggeling, L., and Sahm, H. 1999. L-Glutamate and L-Lysine: Traditional products with impetuous developments. Appl. Micobiol. Biotechnol. 52: 146-153.

Eggeling, L., Oberle, S., and Sahm, H. 1998. Improved L-lysine yield with *Corynebacterium glutamicum*: use of dapA resulting in increases flux combined with growth limitation. Appl. Microbiol. Biotechnol. 49: 24-30.

Eikmanns, B. J., Follettie M. T., Griot, M.U., and Sinskey A.J. 1989. The phosphoenolpyruvate carboxylase gene of *Corynebacterium glutamicum*: molecular cloning, nucleotide sequence, and expression. Mol. Gen. Genet. 218: 330-339.

Eikmanns, B. J., Metzger, M., Reinscheid D.J., and Sahm, H. 1991. Amplification of three threonine biosynthesis genes in *Corynebacterium glutamicum* and its influence on carbon flux in different strains. Appl. Microbiol. Biotechnol. 102: 93-98.

Eikmanns, B. J., Thum-Schmitz N., Eggeling, L., Lüdtke, K., and Sahm, H. 1994. Nucleotide sequence, expression, and transcriptional analysis of the *Corynebacterium glutamicum gltA* gene encoding citrate synthase. Microbiol. 140: 1817-1828.

Gubler, M., Park, S.M., Jetten, M., Stephanopoulos, G., and Sinskey, A.J. 1994. Effects of phosphoenolpyruvate carboxylase deficiency on metabolism and lysine production in *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. 40:857-863.

Gutmann, M., Hoischen, C., and Krämer, R. 1992. Carrier-mediated glutamate secretion by *Corynebacterium glutamicum* under biotin limitation. Biochim. Biophys. Acta 1112: 115- 123.

Hanahan, D. 1985. Techniques for transformation of *E. coli*. In DNA cloning I. D.M. Glover ed. IRL Press, Oxford, Washington DC. p. 109-135.

Hoischen, C., and Krämer, R. 1990. Membrane alteration is necessary but not sufficient for effective glutamate secretion in *Corynebacterium glutamicum*. J. Bacteriol. 172: 3409- 3416.

Ikeda, M. and Katsumata, R. 1993. Metabolic engineering to produce tyrosine or phenylalanine in a tryptophan-producing *Corynebacterium glutamicum* strain. Appl. Environm. Microbiol. 58: 781-785.

Katsumata, R., and Ikeda, M. 1993. Hyperproduction of tryptophan in *Corynebacterium glutamicum* by pathway engineering. Bio/Technology 11: 921-925.

Krämer, R. 1996. Genetic and physiological approaches for the production of amino acids. J. Biotechnol. 45: 1-21.

Leuchtenberger, W. 1996. Amino acids - Technical production and use. In: Biotechnology, vol. 6. H.-J. Rehm, G. Reed, A. Pühler, P. Stadler, and M. Roehr, eds. VCH, Weinheim. p. 465-502.

Liebl, W., Bayerl, A., Schein, B., Stillner, U, and Schleifer, K.-H. 1989. High efficiency electroporation of intact *Corynebacterium glutamicum* cells. FEMS Microbiol. Lett. 65: 299-304.

Marx, A., de Graaf, A. A., Wiechert, W., Eggeling, L., and Sahm, H. 1996. Determination of the fluxes in the central metabolism of *Corynebacterium glutamicum* by nuclear magnetic resonance spectroscopy combined with metabolite balancing. Biotechnol. Bioeng. 49: 111-129.

Marx, A., Striegel, K., de Graaf, A. A., Sahm, H., and Eggeling, L. 1997. Response of the central metabolism of *Corynebacterium glutamicum* to different flux burdens. Biotechnol. Bioeng. 56: 168-180.

Menkel, E., Thierbach, G., Eggeling, L. and Sahm, H. 1989. Influence of increased aspartate availability on lysine formation by a recombinant strain of *Corynebacterium glutamicum* and utilization of fumarate. Appl. Environm. Microbiol. 55: 684-688.

Momose, H., and Tagaki, T. 1978. Glutamic acid production in biotin-rich media by temperature sensitive mutants of Brevibacterium lactofermentum, a novel fermentation process. Agric. Biol. Chem. 10: 1911-1917.

Morbach, S., Sahm, H., and Eggeling, L. (1995). Use of feedback-resistant threonine dehydratases of *Corynebacterium glutamicum* to increase carbon flux towards L- isoleucine. Appl. Envirionm. Microbiol. 61: 4315-4320.

Nunheimer, T.D., Birnbaum, J., Ihnen, D., and Demain, A.L. 1970. Product inhibition of the formation of glutamic acid. Appl. Microbiol. 20: 215-217.

Ozaki, H., and Shiio, I. 1969. Regulation of the TCA and glyoxylate cycles in *Brevibacterium flavum*. J. Biochem. 66: 297-311.

Palmieri, L., Berns, D., Krämer, R., and Eikmanns, M. 1996. Threonine diffusion and threonine transport in *Corynebacterium glutamicum* and their role in threonine production. Arch. Microbiol. 165: 48-54.

Park, S.M., Shaw-Reid, C., Sinskey, A.J., and Stephanopoulos, G. 1997. Elucidation of anaplerotic pathways in *Corynebacterium glutamicum* via ^{13}C-NMR spectroscopy and GC-MS. Appl. Microbiol. Biotechnol. 47: 430-440.

Petersen, S., de Graaf, A.A., Eggeling, L., Möllney, M., Wiechert, W., and Sahm, H. 2000. *In vivo* quantification of parallel and bidirectional fluxes in the anaplerosis of *Corynebacterium glutamicum*. J. Biol. Chem. 275: 35932-35941.

Peters-Wendisch, P. G., Eikmanns, B. J., Thierbach, G., Bachmann, B., and Sahm,. H. 1993. Phosphoenolpyruvate carboxylase in *Corynebacterium glutamicum* is dispensable for growth and lysine production. FEMS Microbiol. Lettters 112: 269-274.

Peters-Wendisch, P. G., Kreutzer, C., Kalinowski, J., Patek, M., Sahm, H., and Eikmanns, B. J. 1998. Pyruvate carboxylase from *Corynebacterium glutamicum*: characterization, expression and inactivation of the *pyc* gene. Microbiol. 144: 915-27

Peters-Wendisch, P. G., Wendisch, V. F., de Graaf, A. A., Eikmanns, B. J., and Sahm, H. 1996. C_3-Carboxylation as an anaplerotic reaction in phosphoenolpyruvate carboxylase-deficient *Corynebacterium glutamicum*. Arch. Microbiol. 165: 387-396.

Peters-Wendisch, P. G., Wendisch, V. F., Paul, S. Eikmanns, B. J., and Sahm, H. 1997. Pyruvate carboxylase as an anaplerotic enzyme in *Corynebacterium glutamicum*. Microbiol. 143: 1095-1103.

Reinscheid, D.J., Kronemeyer, W., Eggeling, L., Eikmanns, B.J., and Sahm, H. 1994. Stable expression of hom-1thrB in *Corynebacterium glutamicum* and its effect on the carbon flux to threonine and related amino acids. Appl. Environm. Microbiol. 60: 126-132.

Sahm, H., Eggeling, L., Eikmanns, B., and Krämer, R. 1995. Metabolic design in amino acid-producing bacterium *Corynebacterium glutamicum*. FEMS Microbiol. Rev. 16:243-252.

Sambrook, J., Fritsch, E. F., and Maniatis,T. 1989. Molecular cloning: A laboratory manual. Cold Spring Habor Laboratory Press, Cold Spring Habor, New York.

Schrumpf, B., Schwarzer, A., Kalinowski, J., Pühler, A., Eggeling, L., and Sahm, H. 1991. A functional split pathway for lysine synthesis in *Corynebacterium glutamicum*. J. Bacteriol. 173: 4510-4516.

Shiio, I., Otsuka, S.I., and Takahashi, M. 1962. Effect of biotin on the bacterial formation of glutamic acid. I. Glutamate formation and cellular permeability of amino acids. J. Biochem. 51: 56-62.

Stephanopoulos, G., and Vallino, J.J. 1991. Network rigidity and metabolic engineering in metabolite overproduction. Science 252: 1675-1681.

Takinami, K., Yoshii, H., Tsuri, H., and Okada, H. 1965. Biochemical effects of fatty acids and its derivatives on L-glutamic acid fermentation. III. Biotin-Tween 60 relationship in the accumulation of L-glutamic acid and the growth of *Brevibacterium lactofermentum*. Agr. Biol. Chem. 29: 351-359.

Tosaka, O., Morioka, H., and Takinami, K. 1979 The role of Biotin-dependent pyruvate carboxylase in L-lysine production. Agric. Biol. Chem. 43: 1513-1519.

Vallino, J.J., and Stephanopoulos, G. 1993. Metabolic flux distributions in *Corynebacterium glutamicum* during growth and lysine overproduction. Biotechnol. Bioeng. 41: 633-646.

Vrljic, M., Sahm, H., and Eggeling, L. 1996. A new type of transporter with a new type of cellular function: L-lysine export from *Corynebacterium glutamicum*. Mol. Microbiol. 22: 815-826.

Vrljic, M., Garg, J., Bellmann, A., Wachi, S., Freudl, R., Malecki, M.J., Sahm, H., Kozina, V.J., Eggeling, L., and Saier, M.H. Jr. 1999. The LysE superfamily: Topology of the lysine exporter LysE of *Corynebacterium glutamicum*, a paradyme for a novel superfamily of transmembrane solute translocators. J. Mol. Microbiol. Biotechnol. 1: 327-336.

Wendisch, V., de Graaf, A.A., Sahm, H., and Eikmanns, B.J. 2000. Quantitative determination of metabolic fluxes during coutilization of two carbon sources: Comparative analyses with *Corynebacterium glutamicum* during growth on acetate and/or glucose. J. Bacteriol. 182: 3088-3096.